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RESEARCH ARTICLE

Outcrossing rates in two seed orchards of *Pinus nigra* Arn. in Bulgaria

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Abstract

Seed orchards are an important source of seeds with improved genetic quality. Here we present results of a study on the genetic composition of seed orchards crop of Austrian black pine (*Pinus nigra* Arn.). Two seed orchards were included in the study – a clonal seed orchard established near Sliven, and a seedling seed orchard, established in the region of Simitli. The outcrossing rates were higher in the clonal seed orchard (Sliven), 0.873 and 0.806 multi-locus (t_m) and single-locus (t_s) estimates, respectively. The same values were 0.623 and 0.530, respectively, in the seedling seed orchard (Simitli). The inbreeding coefficient was positive in both cases, but was significantly different from zero in the clonal seed orchard only (0.101 vs. 0.032). Thus, the two studied seed orchards demonstrate different genetic efficiency. The results are discussed in relation to seed orchard management and efficiency.

Keywords

ex situ conservation, genetic efficiency, outcrossing rates, inbreeding, seed production

Introduction

The seed orchards are one of the tools for increasing of productivity and adaptation of the newly established plantations, and have, at the same time, an important role in *ex situ* conservation of forest genetic resources (Gagov, 2005; Alexandrov, Dobrev, 2012). Besides their primary function of producing seeds, the orchards could be used for other purposes related to tree breeding, such as controlled crossing, studying the flowering

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process, testing the combining ability and breeding values of the clones/genotypes. More than 50 seed orchards for 12 species have been established in Bulgaria during the last five decades, predominantly for conifers, with a few exceptions (Anonymous, 2023).

The aim when establishing a seed orchard is to bring together selected genotypes, usually not related plus trees, often from different populations, and to expect genetic improvement in the progeny as a result of mating among the trees included in the orchard. Bringing together superior trees can be done either directly – by cloning these trees, in most cases by grafting, or indirectly – by raising their progeny. In the first case the result is a clonal seed orchard (CSO) and in the second one – a seedling seed orchard (SSO) (Gagov, Dobrinov, 1987; Funda, El-Kassaby, 2012). Although the two types have their advantages and disadvantages, the clonal ones are preferred because of the larger genetic gain, and especially in conifers, for the early starting of flowering and cone setting (Funda, El-Kassaby, 2012). However, the seedling seed orchards are also considered an important seed source, especially when there is a shortage of seeds for afforestation (Kowalczyk, 2008; Rosvall, Lindgren, 2012).

The flowering and seed production in the seed orchards of many tree species were studied extensively, including in Bulgaria. (Dobrinov, Gagov, 1986; Kang, Lindgren, 1998, 1999; Kang et al., 2001, 2003; Gömöry et al., 2003). The seed orchards of *Pinus nigra* in particular received considerable attention, especially within the area of the species' natural distribution (Matziris, 1993, 1994, 2002; Climent et al., 1997; Bilir et al., 2002; Alizoti et al., 2008; Sivacioglu, Ayan, 2010; Yıldız, Özel, 2019) but also in some other regions where the Austrian pine is considered economically important (Machanska et al., 2013).

More than 10 seed orchards of *P. nigra* of both types – clonal and seedling – were established in Bulgaria, and in most of them the genetic diversity in the seed orchard crops had not been evaluated. Zhelev et al. (2010) performed a pilot study in a clonal seed orchard established near Sliven and found relatively high diversity in the progeny, with multilocus outcrossing rate 0.952. Some phenological observations were performed indicating a good flowering synchrony among the clones in the seed orchard (Nedkova 2005), but generally, no complete study on the fertility variation and genetic diversity of the seed orchard crops was performed to date.

Large-scale study on the seed orchards of coniferous species established in Bulgaria was launched in order to fill in this gap. The present study represents an early stage of this initiative. Its main objective was to evaluate the genetic composition and mating system in two seed orchards of *Pinus nigra* in Bulgaria. The two target orchards were of different types – one clonal and one seedling seed orchard, which provided an opportunity to compare their genetic efficiency.

Material and methods

Two seed orchards of different types were studied – a seedling and a clonal seed orchard. The former is situated near Sliven, at about 215 m altitude, and geographic coordinates 42.67038 N, 26.42611 E. It was established in the period 1990-1992 and

contains a total of 152 clones from Bulgaria, Greece and North Macedonia (Gagov, 1992). The seedling seed orchard is situated in the region of Simitli, on a mountain slope, at altitude 1100-1130 m, and geographic coordinates 41.8983 N, 23.2430 E. It was established in 1986 by planting seedlings originating from a local natural stand. Top pruning was applied in both orchards as a measure of crown management for easier cone collection. Cones were harvested from 31 individuals in the seedling seed orchard and 47 individuals in the clonal seed orchards. The relatedness among individuals in the former is not known, while in the latter, each individual belonged to a different clone. Seeds were extracted from the cones and kept individually in a refrigerator before the analysis.

Seeds germinating on moist filter paper in petri dishes were used for the isozyme analysis. Seven seeds per individual were analyzed. Haploid endosperms and diploid embryos of the seeds were homogenized separately in Tris-HCl buffer pH 7.3 and the homogenates were transferred into the gels by means of Whatmann 3MM chromatographic paper wicks 3x8 mm.

Standard 12% starch gel electrophoresis in two buffer systems (Table 1) was applied to separate the isoenzyme variants. Four enzyme systems were studied providing scorable and interpretable results. One locus of each enzyme system was used for the analysis (Table 1). Gel staining was done following Cheliak, Pitel (1984) and Conkle et al. (1982).

Maternal genotypes were identified based on seven endosperms, which reduced the probability of misidentifying of a diploid genotype when studying haploid endosperms to 0.016, because this probability is defined as 0.5^{n-1}, where n is number of the analyzed endosperms. Diploid genotypes of the progeny were scored directly from electrophoregrams.

Outcrossing rates and other mating system parameters were calculated using the mixed mating model of Ritland (2002) with the software MLTR for Windows, version 2.4. (Ritland, 2002). The following parameters were estimated: allele frequencies in pollen cloud and in ovules, multilocus (t_m) and single-locus (t_s) estimates of outcrossing rates, inbreeding coefficient of maternal parents (F), the correlation of outcrossed paternity (r_p), and correlation of t-estimate (r_t). The standard errors estimates were based on 100 bootstraps.

Table 1. Enzyme and buffer systems studied, and loci scored

Enzyme system (abbreviation, E.C. code *)	Locus	Buffer system**
Glutamate-oxaloacetate transaminase (GOT, 2.6.1.1)	Got-2	A
Menadion reductase (MNR, 1.6.99.2)	Mnr-1	A
Phospho-glucose isomerase (PGI, 5.3.1.9)	Pgi-2	A
6-phosphogluconate dehydrogenase (6PGD, 1.1.1.44)	6-Pg-2	TC

* E.C. – Enzyme Commission

** A – Ashton buffer system, pH 8.1 (Ashton, Braden, 1961); TC – Tris Citrate buffer system, pH 7.0 (Shaw, Prasad, 1970)

Results and discussion

Allele frequencies estimated in the pollen cloud and in ovules are presented in Table 2. Three alleles were found in all loci, but not in all cases. Alleles Pgi-2-3 and 6pg2-3 were found only in the pollen cloud of both orchards, alleles Mnr1-3 and Got2-3 – in the pollen clouds of CSO Sliven and SSO Simitli, respectively. The differences among the allele frequencies in the two orchards were of small magnitude ranging from 0.003 to 0.139 for the pollen cloud and from 0 to 0.165 for the ovules.

Table 2. Estimated allele frequencies in the pollen and in the ovules in the studied seed orchards

Locus	Allele	CSO Sliven		SSO Simitli	
		Pollen	Ovules	Pollen	Ovules
Pgi2	1	0.416	0.362	0.466	0.371
	2	0.577	0.638	0.527	0.629
	3	0.007	0.000	0.008	0.000
Mnr1	1	0.382	0.362	0.419	0.500
	2	0.614	0.638	0.581	0.500
	3	0.004	0.000	0.000	0.000
Got2	1	0.473	0.351	0.589	0.516
	2	0.527	0.649	0.388	0.484
	3	0.000	0.000	0.023	0.000
6pg2	1	0.367	0.234	0.375	0.355
	2	0.615	0.766	0.610	0.645
	3	0.018	0.000	0.015	0.000

Higher outcrossing rates were found in Sliven CSO – multilocus estimate t_m was 0.873 and the single-locus estimate was 0.806. The same parameters in the Simitli SSO were substantially lower – 0.623 and 0.530, respectively. These results indicate that about 88% of the seed crops in CSO Sliven resulted from outcrossing, while in SSO Simitli the proportion of outcrossed seeds was only 62%. There are several plausible explanations of the observed results. The first one is related to the age of the orchards – it is comparable, with some 5-6 years of difference between their establishment, but the age when the individuals of seed origin start to produce cones and male strobili is considerably higher than that of the individuals resulting from grafting (Funda, El-Kassaby, 2012; Rosvall, Lindgren, 2012). Therefore, it is expected that not all individuals in the SSO Simitli started to produce reproductive organs at the time of pollination that resulted in the studied cones and seeds. The flowering in the CSO Sliven started a few years after the successful grafting, and the analysis of 2003 seed orchard crops revealed outcrossing rates even higher than those found in the present study (Zhelev et al., 2010). The second factor contributing to the higher outcrossing rates in Sliven CSO is the more favorable climate conditions on the site (~200 m altitude)

in comparison to the much harsher climate conditions in Simitli SSO (~1100 m). Late frosts could negatively affect the pollination and could contribute substantially to unequal contribution of individuals to seed orchard crops. The third factor could be the background pollen contamination, which is a detrimental factor affecting the genetic diversity and adaptation of seed orchard crops. The issue had been studied extensively in many conifers (El-Kassaby et al., 1989; Slavov et al., 2005; Kaya et al., 2006). Simitli SSO is surrounded by natural stands of different species, including *P. nigra*, and, therefore, background pollen contamination is quite a probable event in this seed orchard.

Table 3. Outcrossing rates, inbreeding and correlations in the studied seed orchards (standard deviations in parentheses)

Parameters	CSO Sliven	SSO Simitli
F	0.101 (0.088)	0.032 (0.1)
tm	0.873 (0.038)	0.623 (0.043)
-ts	0.806 (0.054)	0.530 (0.035)
tm-ts	0.067 (0.030)	0.093 (0.027)
rp (multilocus estimate)	0.037 (0.052)	0.095 (0.096)
rt	0.168 (0.135)	-0.014 (0.052)

Legend: F – inbreeding coefficient of maternal parents, t_m – outcrossing rate, multilocus estimate; t_s – outcrossing rate, single-locus estimate, r_p – correlation of outcrossed paternity, r_t – correlation of t-estimate.

The obtained outcrossing rates are well within the range of these recorded for other Pine species. Zhelev et al. (2010) reported higher values for the same CSO in Sliven – 0.952 multilocus estimate and 0.845 single-locus estimate. The same values were even higher for a natural stand of the same species – 0.965 and 0.870, respectively. Similar values were reported for another two-needle pines – in *P. leucodermis* natural populations outcrossing rates (t_m and t_s) varied from 0.735 to 0.840 (Morgante et al., 1991), in seed orchards of *P. sylvestris* – 0.98 (Muona, Harju, 1989; El-Kassaby et al., 1989). In most coniferous species, the outcrossing rates tend to be high, usually higher than 0.8, and often higher than 0.9, but in almost all studies, some percent of inbreeding was detected. Çengel et al. (2012) found an excess of heterozygotes in the Turkish populations of *P. nigra* subsp. *pallasiana*, which resulted in negative estimates of the inbreeding coefficient, but they used different types of genetic markers. Differences between t_m and t_s were of magnitude up to 0.1. Such differences could be due to gametic disequilibrium (Yeh, Morgan, 1987) or to violation of the model assumptions (Ritland, 2002). When single-locus selfing rate is higher than the multi-locus one, as it was in the present study, this suggests mating among relatives, or biparental inbreeding. Inbreeding coefficient was 0.101 in Sliven CSO and 0.032 in Simitli SSO, and only in the former case it was significantly different from zero.

The correlation parameters are more difficult to interpret because they are more sensitive to the violations of the model assumptions (Ritland, 2002). The correlation of outcrossed paternity, or the probability that a randomly chosen pair of progenies from the same array are full sibs, was low – 0.037 (CSO Sliven) and 0.095 (SSO Simitli).

If the results are translated into more practical language, they indicate that about 88% of the seeds in the crop of CSO Sliven are due to outcrossing, but this also means that a minimum of 12% of the progeny is due to self-fertilization. In Simitli SSO these ratios were 63% outcrosses and 37% inbred seeds (Table 3).

The results of this study could be useful for seed orchard management. Outcrossing rates of CSO Sliven estimated in this study are lower than these reported in a previous one (Zhelev et al., 2010). Possible reasons could be, first, fluctuations in different years due to the environmental conditions during the pollination process, and second, dropping out of about one-half of individuals in the orchard, as a result of a fire event. However, due to the fact that each clone was represented by 5 to 10 ramets, the last inventory revealed that all clones are still represented in the orchard and there are good prospects for its restoration.

One real obstacle is the lack of proper management of the established seed orchards. Still, there is only one next-generation seed orchard in Bulgaria, of *Abies alba* (Gagov, 2005), and there are no plans for next-generation seed orchards for pine species. There is a lot of experience in this respect (Funda, El-Kassaby, 2012) and proper designs developed (Lstibůrek, El-Kassaby, 2010). Another issue related to seed orchards in Bulgaria is their underutilization. According to the Annual estimate (Anonymous, 2023), the need of *P. nigra* seeds for 2022/2023 is about 215 kg, which quality in a good year could be easily met by the numerous clonal and seedling seed orchards of the species established in the country. Evidently, the importance of the seed orchards as a valuable source of genetically improved seeds still has to be properly evaluated and promoted.

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